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Functional regulation of the human integrin VLA-1 (CD49a/CD29) by divalent cations and stimulatory β1 antibodies

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Abstract

We have investigated the regulation by divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} of the functional activity of the human integrin VLA-1 expressed on neuroblastoma NB100 cells. VLA-1-mediated adhesion of NB100 cells to ligand collagen type I was supported by either mM concentrations of extracellular Mg^{2+} or μM levels of Mn^{2+}. In contrast, Ca^{2+} alone did not induce activation of VLA-1 but exerted a potent inhibitory effect on the Mg^{2+}-supported cell adhesion. We have also demonstrated that VLA-1 can be directly activated by the stimulatory monoclonal antibody TS2/16 specific for the integrin β1 subunit, resulting in effective adhesion of NB100 cells to type I collagen. This study has been possible by using a novel blocking VLA-α1 specific monoclonal antibody, 5E8D9.

Key words: Cell adhesion; VLA integrin; Divalent cation

1. Introduction

Integrins are a family of heterodimeric α/β cell surface adhesion receptors that mediate interactions of cells with components of the extracellular matrix (ECM) or with other cells (see [1–4] for recent reviews). All α and β subunits of integrins share a number of common structural features. The integrin β subunits are transmembrane glycoproteins with a large extracellular domain containing a three-fold repeat of a cysteine rich segment and a 100–200 amino acid highly conserved region. The integrin α subunits are also transmembrane glycoproteins with a long extracellular region which contains in the N-terminal part seven homologous domains of approximately 60 residues. Three or four of the seven homologous domains are putative divalent cation binding sites which show sequences with homology to the Ca^{2+} binding proteins calmodulin, parvalbumin and troponin C. Consistent with this is the absolute requirement for divalent cations of integrins to mediate effective interaction with ligands.

One group within the integrins is the VLA subfamily containing at least nine members, each with a distinct α subunit non-covalently associated with the common β1 subunit [3,5,6]. Most VLA integrins seem to function essentially as cellular receptors for ECM proteins such as collagen, laminin and fibronectin. However, VLA-4 besides acting as a cellular receptor for fibronectin also mediates the interaction with a cellular ligand, VCAM-1, expressed on cytokine-activated endothelial cells [7] and has been implicated in homotypic cell interactions as well [8]. Recently a role for VLA-2 and VLA-3 in cell-cell interactions has also been suggested [9–10].

The divalent cation requirements for specificity and affinity of ligand binding have been recently described for integrins VLA-2, VLA-3, VLA-4 and VLA-6 [11–17]. An emerging general feature in the mechanism by which different divalent cations regulate functional activity of these VLA integrins is that both Mg^{2+} at concentrations in the mM range and Mn^{2+} in the μM range induce an active state resulting in effective binding to ligands. Conversely, Ca^{2+} generally exerts inhibitory effects on the function of VLA integrins. These regulatory mechanisms of integrin activation are not restricted to the VLA (β1) subfamily since the interactions of the leukocyte (β2) and β3 integrins seem to be regulated by divalent cations in a similar manner [18–21].

Functional activation of integrins can also be experimentally induced from outside the cells with stimulating mAbs specific for either the α or the β subunit which impose an integrin conformation favorable for interaction with ligands [22–27]. Activation of different VLA integrins can be achieved with mAb specific for the β1 subunit [24–27]. These mAb induce interaction of VLA-2, VLA-4, VLA-5 and VLA-6 with ECM proteins COLL, FN and LM as well as interaction of VLA-4 with the cellular ligand VCAM-1 [11–13,24–27].

Whereas, as mentioned above, the regulation of function of VLA-2, VLA-3, VLA-4, VLA-5 and VLA-6 has been characterized, very few studies on the control of functional activity of VLA-1 have been reported so far, probably due to the limited availability of anti-VLA-1

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Abbreviations: mAb, monoclonal antibody; ECM, extracellular cell matrix; COLL, collagen; LM, laminin; FN, fibronectin; IL-2, interleukin 2.

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mAbs that block the interaction of this integrin with ligands. In this report, we describe the regulation of the functional activity of VLA-1 expressed on a neuroblastoma cell line by divalent cations and stimulatory anti-β1 mAb TS2/16 by using a novel blocking VLA-α1 specific mAb.

2. Materials and methods

2.1. Cells and cell cultures

The neuroblastoma cell line NB100 and the melanoma cell line DX3 were obtained from the Imperial Cancer Research Fund (London, UK). These two cell lines were grown in RPMI medium supplemented with 10% FCS (Biowhittaker, Fontenay Sous Bois, France), 50 IU/ml penicillin, 50 μg/ml streptomycin (ICN Biomedicals, Costa Mesa, CA).

2.2. Antibodies

The following mAb were obtained in our laboratory and described elsewhere: HC1/1 (CD11c) [28], D3/9 (CD45) [29], Lia 3/2 (CD18) [30], Lia 1/2 (CD29) [9], HP2/1 (CD49d) [9]. mAb Tea 1/41 (CD49b) was obtained in our laboratory and will be described elsewhere. The mAb TS2/7 (CD49a) [31], TS2/16 (CD29) [31], 12F1 (CD49b) [32], P1B5 (CD49c) [33], GoH3 (CD49f) [34] have been previously described.

2.3. Affinity chromatography purification of β1 integrins

For purification of total β1 integrins an immunoaffinity chromatography column was prepared by covalently coupling purified anti-β1 mAb TS2/16 (at 2 mg/ml) to 3 ml of CNBr-activated CL-4B Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) following the manufacturer's instructions.

A mixture of β1 integrins was obtained from Triton X-100 lysates of normal human spleen. Spleen (50 g) was diced, sieved and lysed in 300 ml of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM $MgCl_2$, 200 μM $MnCl_2$, 1% Triton X-100, 0.02% NaN_3, 1 mM PMSF, 0.2 units/ml aprotinin and 5 mM iodoacetamide, pH 8.0) for 2 h at 4°C. The cell lysate was centrifuged at 3,000 × g for 30 min at 4°C and then ultracentrifuged at 100,000 × g for 1 h at 4°C. The lysate was precleared by passing through a 2 ml pre-column of glycine-Sepharose CL-4B (pre-equilibrated in lysis buffer) and then loaded onto the 3 ml column of TS2/16-Sepharose CL-4B (pre-equilibrated in lysis buffer) at a flow rate of 0.5 ml per min. The column was then washed sequentially with 15 ml of lysis buffer and 15 ml of washing buffer (50 mM ethanolamine, 0.2% Triton X-100, 0.5 M NaCl, 2 mM $MgCl_2$, 200 μM $MnCl_2$, 1 mM PMSF, pH 10.0) and bound β1 integrins were finally eluted with 50 mM ethanolamine, 0.5 M NaCl, 2 mM $MgCl_2$, 200 μM $MnCl_2$, 1% octylglucoside, 1 mM PMSF, pH 12.0 at a flow rate of 0.5 ml per min. Fractions of 0.5 ml were collected and neutralized with 0.1 volume of 1 mM Tris, pH 6.7. Fractions containing β1 integrins were identified by SDS-8% PAGE followed by silver staining. The yield of total β1 integrins was estimated to be approximately 2 mg (in 10 ml of neutralized elution buffer) by comparison of silver staining with known amounts of BSA.

2.4. Obtainment of mAb 5E8D9

Balb/c mice were injected intraperitoneally with approximately 5 μg of affinity chromatography purified β1 integrins on days −48 and −33, and intravenously on day −3. Spleen cells were fused on day 0 with SP2 mouse myeloma cells at a ratio of 4:1 according to standard techniques and distributed in 96-well plates (Costar, Cambridge, MA). After two weeks, hybridoma culture supernatants were harvested and screened for reactivity with purified β1 integrins in an ELISA method and positive hybridomas were subsequently tested for their ability to inhibit the adhesion of the neuroblastoma cell line NB100 to type I collagen and laminin. Hybridomas producing monoclonal antibodies with inhibitory effect on cell adhesion were cloned by limiting dilution. Monoclonal antibody 5E8D9 was selected. This mAb is of the IgG2a subclass.

2.5. Immunoprecipitation

NB100 neuroblastoma and DX3 melanoma cells were iodinated with 0.5 mCi sodium [^{125}I]iodide (ICN Biomedicals, Irvine, Ca) using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen, Sigma, St. Louis, MI) and lysed in phosphate buffered saline (PBS), 1% Triton X-100, 1% haemoglobin, 1 mM PMSF, pH 7.4 for 15 min. For immunoprecipitation, equal amounts of radioactive cell lysates were incubated at 4°C with 100 μl of corresponding hybridoma culture supernatants. After 2 h, 100 μl of culture supernatant containing the rat anti-mouse-IgG mAb 187.1 were added and incubation proceeded for an additional period of 2 h at 4°C. Finally, immunoprecipitates were removed by addition of 30 μl of protein A–Sepharose, incubation for 1 h with continuous stirring and centrifugation at 200 × g followed by processing as previously described [28]. Samples were analyzed on SDS-8% polyacrylamide gels followed by autoradiography with enhancing screens.

2.6. Flow cytometric analysis

Flow cytometry was performed as previously described [19,35]. Briefly, 10^5 cells were incubated for 30 min on ice with 50 μl of culture supernatant or a 1:100 dilution of ascites fluid from the corresponding mAb in each well of a flexiwell plate (Dynatech), washed 3 times with 200 μl of RPMI and incubated for an additional period of 30 min with 50 μl of a 1:100 dilution of FITC-conjugated sheep anti-mouse IgG secondary antibody (Sigma). After appropriate washes with PBS, cells were fixed in 200 μl of 5% formaldehyde in PBS and their fluorescence determined with a FACScan flow cytometer (Becton Dickinson).

2.7. Cell attachment assays

Laminin was a generous gift from Dr. M.A. Lizarbe (Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias Químicas, Universidad Complutense, Madrid). Collagen type I (Vitrogen 100) was purchased from ICN Biomedicals. The adhesion assays were essentially performed as previously described [26]. Briefly, 96-well flat-bottomed plates (Titertek, ICN Biomedicals) were coated overnight at 4°C with either laminin or collagen at 20 μg/ml in PBS. After one wash with 200 μl of PBS, wells were saturated for 1 h at room temperature with boiled 1% BSA in PBS. Plates were then washed three times with PBS and once with 200 μl of HEPES buffer (20 mM HEPES, 150 mM NaCl, 2 mg/ml D-glucose, pH 7.4) and 50 μl of HEPES buffer containing the appropriate dilutions of purified mAb and the chlorides of divalent cations Mg^{2+}/EGTA, Ca^{2+}, and Mn^{2+} or EDTA were added to the wells. Cells (10^5) in 50 μl of HEPES buffer were added to each well and plates were incubated for 45 min at 37°C. The percentage of cells that remained adhered after three washes of wells with 200 μl of HEPES buffer was calculated measuring the absorbance of wells at 540 nm after fixation of adhered cells with 3% formaldehyde in PBS for 10 min and then for 10 min with 2% methanol followed by staining with 0.5% Crystal violet in 20% methanol, washing with water and elution of stain with 0.1 M sodium citrate pH 4.2 in 50% ethanol.

3. Results

The initial selection of hybridomas producing mAb specific for VLA integrins was made based on their reactivity with human purified total β1 integrins immobilized on plastic by an ELISA method (data not shown). For functional selection of mAb specific for VLA integrins, the ELISA-positive hybridoma culture supernatants were subsequently analyzed for their ability to inhibit the adhesion of neuroblastoma (NB100) and melanoma (DX3) cells to the ECM proteins type I collagen and laminin. As shown in Fig. 1, adhesion of NB100 and DX3 cells to these two proteins in medium containing 2 mM Mg^{2+} and 2 mM Ca^{2+} was mediated by β1 integrins since it was completely inhibited by preincubation of the cells with the mAb Lia 1/2 which binds to a functionally relevant epitope on the common β1 subunit [9,26].

We selected the mAb 5E8D9 for its ability to block the adhesion of neuroblastoma NB100 cells to type I colla-

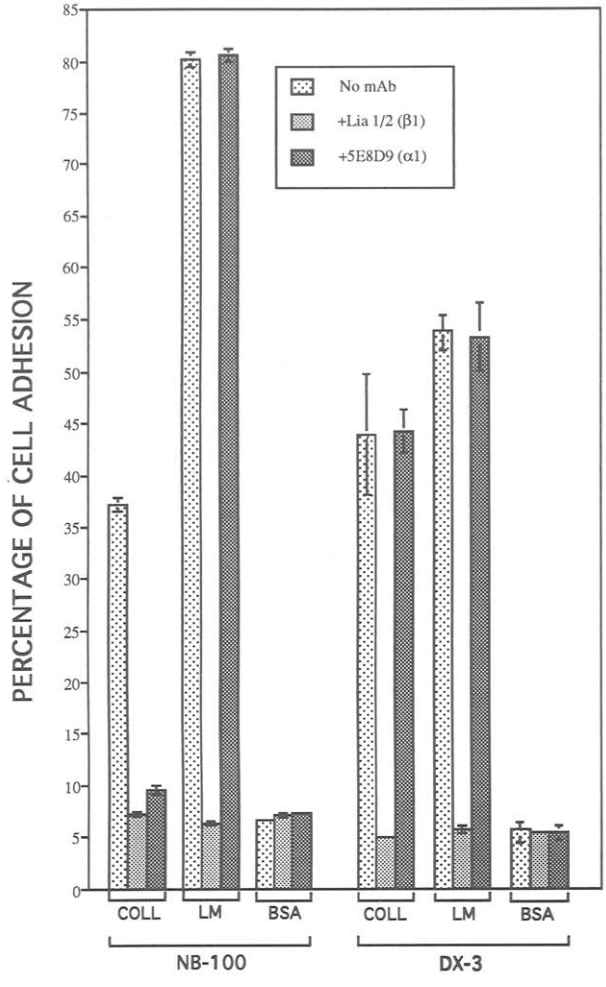

Fig. 1. Adhesion of human neuroblastoma NB100 cells to collagen type I is blocked by anti-VLA-1 mAb 5E8D9. The adhesion of NB100 cells to collagen type I but not to laminin was inhibited by mAb 5E8D9. The adhesion of human melanoma DX3 cells to either collagen or laminin was not affected by mAb 5E8D9. The mAb Lia 1/2 specific for the common β1 subunit of VLA integrins completely blocked the adhesion of both cell lines to either collagen type I or laminin. Cell attachment assay was performed in HEPES buffer containing 2 mM Mg^{2+}, 2 mM Ca^{2+} and 10 μg/ml of corresponding mAb as described in section 2. Data are representative of five independent experiments.

gen (Fig. 1). Interestingly, the adhesion of this cell line to laminin was not affected by mAb 5E8D9. In contrast, the adhesion of the melanoma cell line DX3 to either laminin or type I collagen was unaffected by mAb 5E8D9, suggesting that under the same conditions these two cell types may utilize other different VLA heterodimers for efficient adherence to type I collagen and laminin.

The nature of the molecule recognized by mAb 5E8D9 was determined by immunoprecipitation analysis from lysates of ^{125}I-surface labeled NB100 and DX3 cells and comparison with other VLA heterodimers precipitated with different anti-VLA α and β mAbs. The mAb 5E8D9 immunoprecipitated a 200 kDa subunit associated with the common 130 kDa β1 subunit from both NB100 and DX3 cell lines (Fig. 2). Immunoprecipitation with the control anti-VLA-1 mAb TS2/7 showed that the two bands corresponding to the α1 (CD49a) and β1 (CD29) subunits have exactly the same molecular weight as those obtained with mAb 5E8D9, thus indicating that this mAb is specifically recognizing the α subunit of VLA-1. Also, the pattern of surface staining with the control anti-VLA-1 mAb TS2/7 was identical to that observed with mAb 5E8D9 for all the different types of haemopoietic and non-haemopoietic cells tested, which confirms the specificity of this mAb for the α1 integrin. In contrast, control mAb specific for CD49b, CD49c, CD49d, CD49e and CD49f showed patterns of staining on the different cell types analyzed clearly different to those obtained with mAb 5E8D9 or TS2/7 (data not shown).

As we had found that adhesion of neuroblastoma NB100 cells to type I collagen in medium containing Ca^{2+} and Mg^{2+} was mainly mediated by integrin VLA-1 we characterized in more detail this adhesion system and used it for the study of the regulation by divalent cations of the functional activity of integrin VLA-1. We first analyzed the expression on NB100 and DX3 cells of the VLA heterodimers known to mediate the adhesion of other cell types to collagen or laminin (Fig. 3). VLA-1 (COLL/LM), VLA-2 (COLL/LM) and VLA-3 (FN/

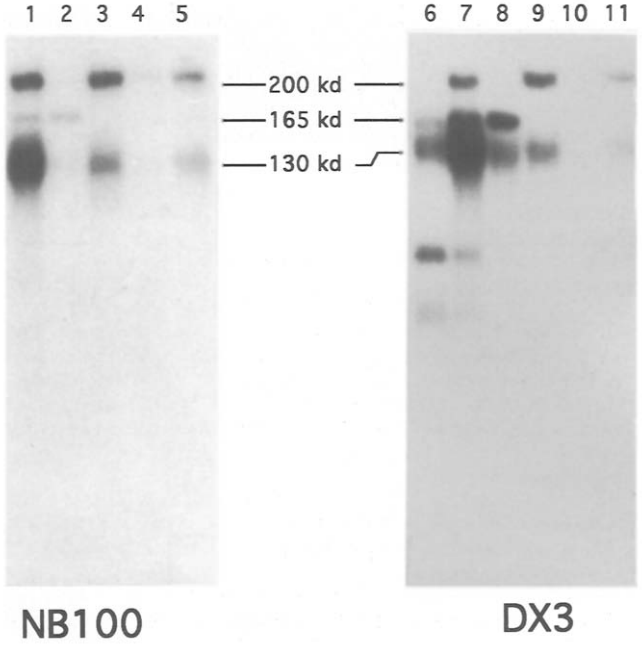

Fig. 2. Immunoprecipitation with mAb 5E8D9 from ^{125}I-labeled lysates of melanoma DX3 and neuroblastoma NB100 cells. mAb 5E8D9 immunoprecipitates two bands of 200 and 130 kDa (lanes 5 and 11) which correspond to the VLA-1 heterodimer as determined by comparison with the control anti-VLA-α1 (CD49a) mAb TS2/7 (lanes 3 and 9). Other control mAb used were: HC1/1 (CD11c) lanes 4 and 10, TS2/16 (CD29) lanes 1 and 7, Tea 1/41 (CD49b) lanes 2 and 8 and HP2/1 (CD49d) lane 6. Immune complexes were isolated, and reduced samples were subjected to SDS-8% PAGE and autoradiography as described in section 2.

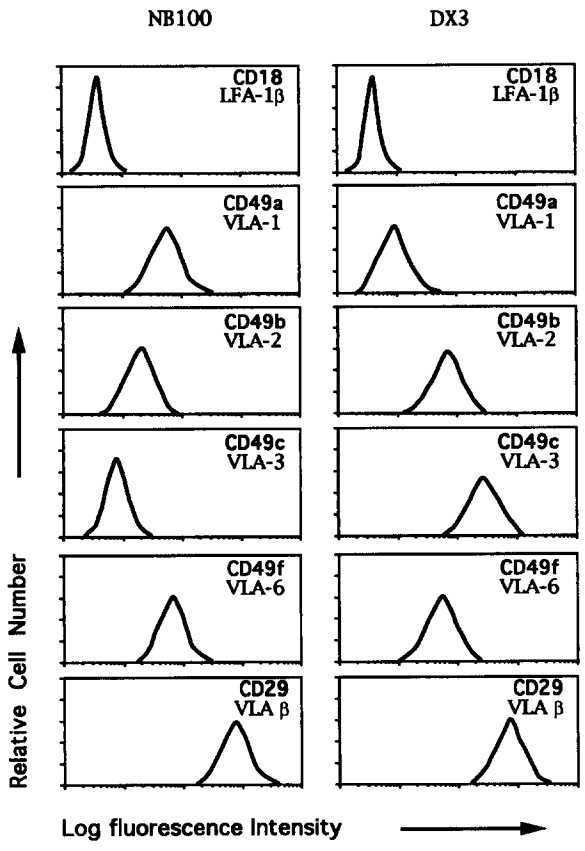

Fig. 3. Cell surface expression of VLA collagen and laminin receptors on neuroblastoma NB100 and melanoma DX3 cells. The level of surface expression of CD18 (negative control), VLA-1 (CD49a), VLA-2 (CD49b), VLA-3 (CD49c), VLA-6 (CD49f) and β1 (CD29) integrin subunits was determined by flow cytometry as described in section 2. MAb used were: Lia 3/2 (CD18), TS2/7 (CD49a), Tea 1/41 (CD49b), P1B5 (CD49c), GoH3 (CD49f).

COLL/LM) α subunits were differentially expressed in NB100 and DX3 cell lines whereas the level of VLA-6 (LM) expression was very similar in both cell lines. Specifically, the level of VLA-1 expression in NB100 cells was clearly higher than in DX3, which would account for the lack of inhibitory effect of mAb 5E8D9 on the adhesion of DX3 to collagen previously observed. Also, comparison of the levels of surface expression among the three VLA integrins known to mediate cell adhesion to collagen showed that on NB100 cells VLA-1 is expressed at a remarkably higher level than VLA-2 or VLA-3, which again could explain the blocking effect of mAb 5E8D9 on the adhesion of this cell line to type I collagen.

We next studied the regulation of functional activity of VLA-1 by divalent cations Ca^{2+}, Mg^{2+} and $Mn^{2+}$by using the interaction of NB100 cells with type I collagen. For both Mg^{2+} and Mn^{2+} the percentage of NB100 cells that adhered to type I collagen increased with the concentration of divalent cation present in the medium (Fig. 4A). At relatively low concentrations of cations, cell adhesion was mainly mediated by VLA-1, as indicated by the potent inhibitory effect that preincubation with mAb 5E8D9 had under all conditions. The level of inhibition of cell adhesion with this mAb was lower at higher concentrations of divalent cations, which may reflect the cation-induced activation of other collagen binding VLA integrins (VLA-2 or VLA-3) on these cells. For all the divalent cation concentrations used, adhesion of NB100 cells to type I collagen was completely dependent on the activity of VLA integrins, as demonstrated by blockade with the anti-β1 mAb Liα1/2. As reported for other integrins [19], the minimal concentration of divalent cation required for effective VLA-1-mediated adhesion was 50-fold higher for Mg^{2+} than for Mn^{2+} suggesting that the later binds to one or several of the three divalent cation binding sites on the CD49a with much higher affinity. On the contrary, Ca^{2+} even at concentrations of up to 5 mM was not able to support the interaction of VLA-1 with type I collagen (not shown). Despite the fact that Ca^{2+} alone did not induce the activation of VLA-1, the question of whether this divalent cation could act as a potentiator of the Mg^{2+}-supported interaction of integrin VLA-1 with ligand still remained open. As shown in Fig. 4B, Ca^{2+} did not exert any potentiating effect on the adhesion induced by Mg^{2+} (at 2 mM) but clearly had a concentration dependent inhibitory effect. As expected, no VLA-1-mediated adhesion of NB100 cells to type I collagen was observed in the absence of divalent cations.

Finally, we investigated whether the integrin VLA-1 on NB100 cells can be directly activated by the anti-β1 mAb TS2/16 resulting in increased adhesion to ligand type I collagen as previously described for VLA-3, VLA-4, VLA-5 and VLA-6 in a number of different cell systems [11–12,26,36]. Fig. 5A shows that mAb TS2/16 effectively augmented the percentage of NB100 cells that adhere to collagen under all the different cation conditions tested. However, this TS2/16-induced increment in cell adhesion to type I collagen due to activation of VLA integrins could only be marginally inhibited with mAb 5E8D9, indicating that other collagen receptors, namely VLA-2 and VLA-3, although expressed at lower levels than VLA-1 on NB100 cells, become also directly activated by mAb TS2/16. Interestingly, in the presence of mAb TS2/16, adhesion of NB100 cells to type I collagen was supported by concentrations of Ca^{2+} in the mM range. However, this Ca^{2+}-supported adhesion was minimally inhibited by mAb 5E8D9, again suggesting that under these conditions of activation of β1 integrins with mAb TS2/16 cell adherence to type I collagen is mediated not only by VLA-1 but also by VLA-2 or/and VLA-3. To determine whether VLA-1 on NB100 cells actually becomes directly activated by mAb TS2/16, we assessed whether the inhibitory effect of mAb 5E8D9 on cell adhesion was more evident in the situation where cell adhesion to collagen is induced in Ca^{2+} with mAb TS2/16 and partially inhibited with an anti-VLA-2 mAb. Under these conditions, the anti-VLA-1 mAb 5E8D9 had a

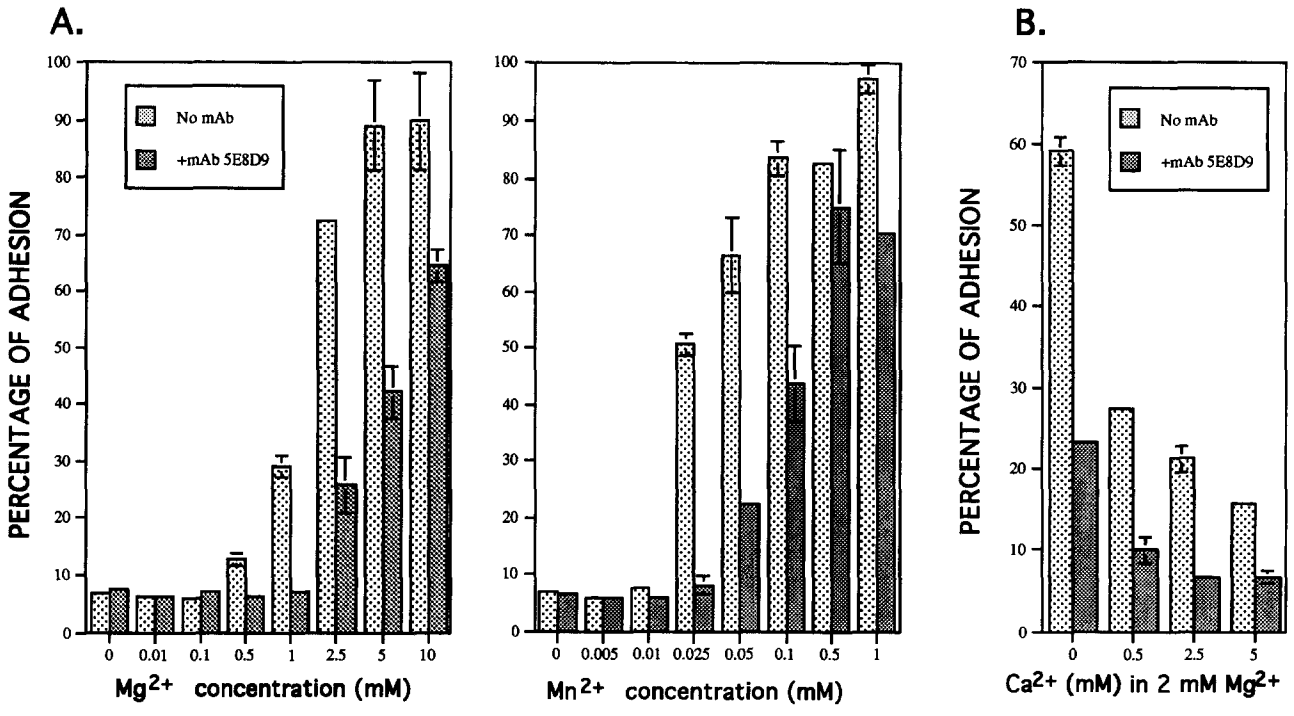

Fig. 4. Divalent cation dependency of the VLA-1-mediated adhesion of human neuroblastoma NB100 cells to collagen type I. (A) Mg^{2+} (in the presence of 1 mM EGTA) at concentrations in the mM range and Mn^{2+} at concentrations in the mM range induce VLA-1-mediated cell adhesion to collagen in a dose dependent manner. (B) Ca^{2+} inhibits the VLA-1-mediated cell adhesion induced by 2 mM Mg^{2+} in a dose-dependent manner. mAb 5E8D9 was used at a final concentration of 10 μg/ml. Cell attachment assays were performed as described in section 2. Data are representative of five different experiments.

clear additive inhibitory effect on cell adhesion indicating that mAb TS2/16 activates not only VLA-2 but also VLA-1 on NB-100 cells (Fig. 5B).

4. Discussion

In this paper we describe the regulation of the functional activity of the integrin VLA-1 (CD29a) by divalent cations Mg^{2+}, Ca^{2+} and Mn^{2+} and also by the stimulatory anti-β1 mAb TS2/16.

We have used the adhesion of the neuroblastoma NB100 cell line to type I collagen as a model system for the study of functional activation of VLA-1. These neuroblastoma cells express on their surface high levels of VLA-1 but only moderate levels of VLA-2 and low levels of VLA-3 and, therefore, constitute an excelent model for analysis of VLA-1 function. This study has been facilitated by the generation of a novel CD49a specific mAb that effectively blocks the interaction of the VLA-1 heterodimer with type I collagen. This anti-VLA-1 mAb was obtained by immunizing mice with a mixture of total β1 integrins purified from human normal spleen. The use of an immunoaffinity column with the stimulating anti-β1 mAb TS2/16 covalently linked to sepharose and the presence of Mn^{2+} and Mg^{2+} throughout the integrin purification and immunization protocols were aimed at preserving β1 integrins in an active conformation able to interact with appropriate ligands. For functional selection of mAbs specific for β1 integrins we assessed their ability to inhibit the adherence of neuroblastoma (NB100) and melanoma (DX3) cell lines to the ECM proteins type I collagen and laminin which are ligands for VLA-1, VLA-2, VLA-3 and VLA-6 integrins. The mAb 5E8D9 was selected for its ability to block the adhesion of NB100 cells to type I collagen. On the contrary, this mAb had no effect on the adhesion of DX3 cells to the same ECM protein. These results indicated that mAb 5E8D9 might be specifically recognizing the a subunit of VLA-1 as neuroblastoma NB100 cells do express a remarkably higher molecular density of VLA-1 on their surface than melanoma DX3 cells and, therefore, the adherence of these two cell lines to collagen was expected to be differentially affected by an anti-VLA-1 mAb. The specificity of mAb 5E8D9 for VLA-1 was confirmed by immunoprecipitation and comparative analysis of the cell surface expression of this molecule with the control anti-VLA-1 mAb TS2/7.

Under physiological divalent cation conditions, i.e. in the presence of extracellular concentrations of Mg^{2+} and Ca^{2+} in the range of 1–2 mM, a significant percentage (35–40%) of NB100 cells adhere to type I collagen and this adhesion is mediated by VLA integrins as demonstrated by complete blocking with the CD29 mAb

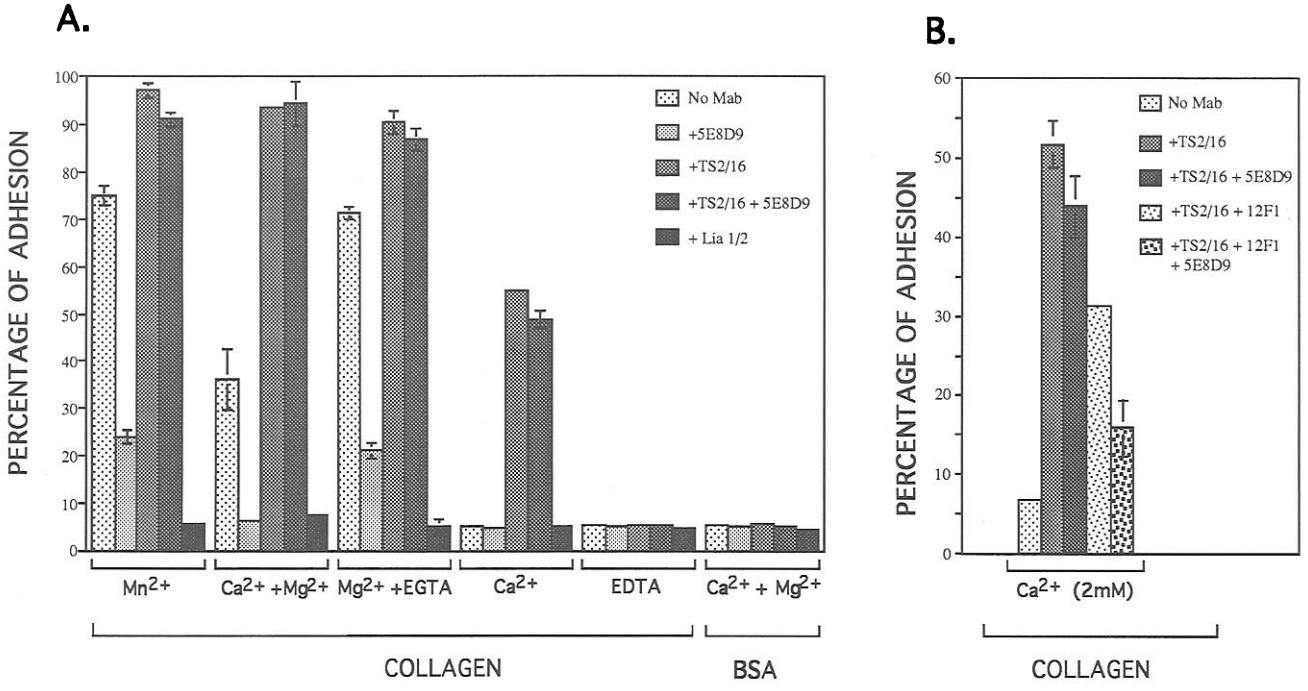

Fig. 5. Stimulatory anti-β1 mAb TS2/16 activates VLA-1 on NB100 cells. (A) mAb TS2/16 induces an important increase in cell adhesion to collagen type I under all divalent cation conditions. No enhancement of cell adhesion to collagen type I in the absence of divalent cation (EDTA) or to bovine serum albumin (BSA) in the presence of Ca^{2+} and Mg^{2+} was induced by mAb TS2/16. (B) TS2/16-induced increment in cell adhesion to collagen type I is inhibited by anti-VLA-1 (5E8D9) and anti-VLA-2 (12F1) mAb. Divalent cation concentrations used were: [Mn^{2+}], 50 μM; [Ca^{2+}+Mg^{2+}], 1 mM Ca^{2+} and 2 mM Mg^{2+}; [Mg^{2+}+EGTA], 2 mM Mg^{2+}/1 mM EGTA; [Ca^{2+}], 2 mM; [EDTA], 2 mM. All mAb were used at a final concentration of 10 μg/ml. Data are representative of five different experiments.

Liα1/2. Using mAb 5E8D9 we have demonstrated that under these cation conditions adhesion of NB100 cells to type I collagen is basically mediated by the integrin VLA-1. The fact that combination of Mg^{2+}/Ca^{2+} supports VLA-1-mediated cell adhesion indicates that under physio-logical conditions VLA-1 is constitutively active and capable of mediating interaction with ligands. This constitutive partial activation of VLA-1 expressed on neuroblastoma NB100 cells clearly contrasts with members of other families of integrins such as the leukocyte integrin LFA-1, where the presence of low concentrations of Ca^{2+} completely inactivate this integrin rendering leukocytes unable to interact with ligand ICAM-1 [18]. This difference in the cation-controlled basal activation state of different integrins may be related to the cell type and has important physiological relevance, as resting leukocytes are normally non-adherent circulating cells that do not interact with other cells or ECM proteins unless they become activated, whereas neuroblastoma or melanoma cells have generally a constitutive adherent phenotype.

Our data also demonstrate that the activity of VLA-1 expressed on human neuroblastoma NB100 cells is controlled by divalent cations Ca^{2+}, Mg^{2+} or Mn^{2+} in a dose-dependent manner. Both Mg^{2+} and Mn^{2+} induce the activation of VLA-1 possibly by imposing a conformation which favours interaction with ligand upon binding to one or more of the three cation binding sites present on the α subunit. Conversely, Ca^{2+} does not support the interaction of VLA-1 with collagen but exerts strong negative effects on the Mg^{2+}-induced activation of this adhesion receptor. These regulatory mechanisms exerted by divalent cations on the activity of VLA-1 are similar to those described for other β1 integrins (VLA3, VLA-4, and VLA-6) as well as integrins belonging to the β2 and β3 families in a number of cell and soluble systems [11–15,17,19–21]. Therefore, general regulatory mechanisms by which divalent cations control the functional activity of integrins are now emerging that span different integrin families.

We have also shown that function of the VLA-1 heterodimer can be regulated through the β1 chain by the specific TS2/16 anti-β1 mAb. Under all the cation conditions tested, mAb TS2/16 increased the percentage of cell adhesion to collagen, reflecting the activation of VLA integrins. However, in the total absence of divalent cations no enhancement in cell adhesion was observed with mAb TS2/16, confirming the absolute requirement for divalent cations of VLA integrins to mediate effective adhesion to ligands. Interestingly, in the presence of mAb TS2/16, mM concentrations of Ca^{2+} are able to support an important percentage of VLA-1 (and VLA-2)-mediated cell adhesion to collagen. This finding of reversion by mAb TS2/16 of the Ca^{2+}-induced inhibition

of adhesion has been also recently described for VLA-3 [10] and indicates that the conformational change induced by mAb TS2/16 in different VLA integrins is not sufficient to mediate effective cell adhesion to ligands unless binding of cation to specific sites on the α subunit occurs. The fact that NB100 cells express, in addition to VLA-1, other collagen receptors on their surface, namely VLA-2 and VLA-3, may explain that the inhibition of cell adhesion observed with mAb 5E8D9 was never complete.

The physiological relevance of cation usage by integrins can be envisioned assuming that extracellular Ca^{2+} maintains the integrins on resting cells in a state of low or basal activation until specific stimuli induce activation of cells altering the conformation of the integrins which in turn results in increased cell adhesion to appropriate ligands. Accordingly, stimulatory mAb such as TS2/16, which induces activation of β1 integrins, or NKI-L16 which induces activation of the β2 integrin LFA-1, are thought to activate integrins by inducing similar conformational changes to those brought about as a consequence of cell activation.

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